Summary

Gram-positive bacteria fulfill a vital role in food and health. Gram-positive bacteria exist as gastroenteric commensals, but also find application in food fermentations and are used as probiotics. Whereas the latter are "generally regarded as safe" (GRAS), also many Gram-positive bacteria are human pathogens.

The mammalian gastrointestinal milieu, which is acidic in nature and enriched in surfactant like antimicrobials, presents a formidable challenge for microbes to colonize it. Hence, the mechanisms used by the Gram-positive organisms to overcome such challenges have been a focus of study in the field of microbiology for quite sometime.

The active efflux of toxic drugs by by transport proteins located in the cytoplasmic bacterial membrane has been known to play a prominent role in the resistance to individual drugs. However, in addition to single or group-specific efflux pumps, bacteria also harbour systems capable of flushing out structurally and functionally distinct toxic molecules in a process called multidrug resistance (3). The MDR phenomenon has been well studied in *Lactococcus lactis* with around 40 putative efflux pumps identified in its genome (2). However, only few have been characterized functionally. *L. lactis* is capable of adapting to structurally diverse synthetic and naturally occuring drugs and surfactants using ATP-binding cassette (ABC) transporter LmrCD as the primary mechanism of defence (2,7). The studies presented in chapter 2 demonstrate that *L. lactis* when deprived of the LmrCD based efflux mechanism can still develop a resistive response against naturally occuring gastrointestinal surfactants, namely the bile acids. Previously, LmrCD was shown to provide protection against the bile acids cholate and the conjugated forms of deoxycholate. On the other hand, cells lacking LmrCD are still able to adapt to high concentrations of cholate, but this resistance cannot be attributed to an active efflux mechanism. DNA micro-array analysis showed no evidence of an altered expression of (putative) membrane transport proteins. Rather, cholate adaptation involved changes in the cell envelop resulting in an altered cell-morphology and a slower gowth

rate. Whereas wild-type *L. lactis* has a typical diplococcal appearance, cholate-adapted cells showed an enhanced flocculation that is governed by the aggregation of long strings of cells, which is reminiscent of free floating biofilms (5). In this respect, bile acids have been shown to promote bacterial biofilm formation (1) suggesting a tentative link between the resistance and the ability to form biofilms. Chapter 3 deals with the phenomenon of cell adherence, biofilm formation and biofilm drug resistance in *L. lactis.* The wild-type and Δ*lmrCD L. lactis* cells are equally capable of forming biofilms on a hydrophobic polystyrene surface under typical growth conditions. In contrast, the slow growing cholate-adapted cells are poor in biofilm formation.

This indicates that adaptation to bile acids has a fitness cost to it. However, in the presence of sub-inhibitory concentrations of cholate, the cholate-adapted cells in particular showed a strong enhancement in biofilm formation. Such biofilms contain a higher number of viable cells that exhibit an enhanced exopolymer production and are highly resistant to bile acids. Contact angle measurements revealed global changes in the physicochemical characteristics of the cell envelop (most likely peptidoglycan macromolecules and other macromolecules at the outer cell surface), which promote a hydrophobic behaviour of the otherwise hydrophilic *L. lactis* surface. This would explain the better adherence of the cholate-adapted cells (when challenged with bile acids) to hydrophobic abiotic surfaces. Bile acids have been shown to stimulate biofilm formation in bacteria via the induction of exopolysaccharides synthesis genes (1). However, our findings showed that this is not the case for all types of bile acids. CLSM imaging revealed that the stimulatory bile acids increase the volume and thickness of the mature biofilm formed by the cholate adapted mutant cells. Taurocholate, the most hydrophilic of all bile acids had the strongest effect on the biofilm matrix volume in a dose dependent manner. However, these studies also show that matrix thickness is not directly related to microbial fitness. The changes in biofilm volume seems to have little effect on the resistance, suggesting that the voids and channels that are formed in the mature biofilm facilitate the entry rather than retarding diffusion of molecules into the biofilm. Taken together the data suggest that the non-transporter based cholate resistance in

L. lactis is due to changes in the cell surface that stimulate cells to form resistant biofilms. On the other hand, the studies also demonstrated that LmrCD plays an important role in lactococcal biofilm resistance. To our knowledge this is the first report that shows that an ABC-type MDR transporter of a Gram-positive micro-organism contributes to biofilm resistance.

The extensive resistance to various bile species left open the possibility of other (putative or known) efflux pumps with a role in the adaptive resistance in *L. lactis*. This question was addressed in chapter 4 where rhodamine 6G, a substrate for LmrCD and some other known lactococcal efflux pumps (4,6) was used as the adaptation agent. Planktonic cells lacking LmrCD readily adapted in small incremental steps. Interestingly, the cells did develop adaptive resistance not only to rhodamine 6G but also to structurally different dyes, drugs and detergents, thus adaptation resulted in the typical MDR phenotype that what not observed with cholate adaptation (Chapter 3). Real time PCR analysis of a screen of putative and known efflux genes suggests that LmrP is involved in this resistance, and possibly some other efflux transporters. Importantly, an unknown MFS type transporter *llmg_0631* was upregulated in the daunomycin exposed resistant cells. The notion that the resistance is partly due to transport also followed from Hoechst 33258 and Rhodamine 6G transport assays. However, unlike the cholate adaptation an alterated biofilm phenotype, no such similar change was detected in the rhodamine 6G adapted cells. In contrast, Rhodamine 6G interfered with cell adherence to abiotic surfaces and it significantly impaired tolerance of mature biofilms of the adapted cells to drugs including rhodamine 6G and daunomycin. Thus, rhodamine resistance was only related to the planktonic phase. The multifactorial nature of this mechanism of resistance was further corroborated by the high susceptibility of one of the mutants to tellurite, an anionic metal which is reduced to elemental tellurium in the cells. Possibly, these cells are equipped with an altered oxidative stress response, a phenomenon that may relate to the mode of action of some of the drugs that causes the formation of oxygen radicals. A detailed investigation of the knockout of putative transporter and LmrP along with their overexpression is

need to fully understand the mechanism of drug resistance in *L. lactis* strains lacking the LmrCD transporter.

Summarizing, we conclude that *L. lactis* has a remarkable ability to resist all kinds of natural and unnatural compounds. Once the first line of defense is inactivated (loss of the major MDR transporter), various specific and less specific backup mechanisms allow the cells to regain resistance, albeit at a fitness cost.

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